# Evaluation of Five Real-Time PCR Assays for Detection of *Mycoplasma pneumoniae*

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**Four commercial real-time PCR assays to detect *Mycoplasma pneumoniae* were tested, and the results were compared with the results for an in-house approach. Despite differences of crossing threshold values of up to 4, assays were able to detect at least 20 CFU/5 μl (52 fg DNA/5 μl) of sample with the Diagenode kit showing the best clinical sensitivity.**

*M*ycoplasma pneumoniae is a common agent of infections of the human respiratory tract ranging from mild cases of tracheobronchitis to severe pneumonia requiring hospitalization of the patient. Furthermore, extrapulmonary manifestations of *M. pneumoniae* infections have been reported (1–3). Especially in epidemic periods, which are reported every 3 to 7 years, up to 40% of community-acquired pneumonia (CAP) cases are caused by *M. pneumoniae* (1, 2), affecting mainly older children but also adults (4, 5). Early and rapid diagnosis of interstitial pneumonia due to *M. pneumoniae* is of importance, since beta-lactam antibiotics are recommended as first-line therapy for CAP and these antibiotics are ineffective against the cell wall-less mycoplasmas. Unfortunately, culture of these bacteria from clinical specimens is time-consuming and has low sensitivity. Serological tests are not helpful in early stages of infection, requiring in many cases paired serum samples for definite interpretation of the results and showing problems with regard to specificity as well as to sensitivity (6, 7). Real-time PCR assays have been developed as a sensitive and specific option for detection of microorganisms in respiratory samples. In the last few years, a broad spectrum of targets for amplification has been reported (8–10). Among these targets, use of copies of repetitive element repMP1 was demonstrated in different studies as a very sensitive approach (9–11). Since a number of variables can influence the results of amplification, there exists the need for inter- and intralaboratory validation of different assays under defined conditions (6). Furthermore, the permanent dynamics on the market of laboratory diagnostics requires testing of real-time PCR kits that are commercially available at a given time. The aim of the present study was to compare the performance of selected commercial real-time PCR assays with a characterized in-house approach on a set of standardized samples of different origin.

*M. pneumoniae* strain M129 (ATCC 29342) was grown in cell culture flasks with PPLO medium (Becton Dickinson, Sparks, MD, USA) at 37°C. After the color of the medium changed, attached cells were washed twice with phosphate-buffered saline (PBS) and harvested using a cell scraper.

Four commercially available PCR kits were included in the study, and the results were compared to the in-house approach for amplifying the repMP1 copies as a multicopy target (11). Of the 14 repMP1 elements scattered all over the genome of *M. pneumoniae* (12), amplification of at least 10 copies can be expected. To limit the influence of different platforms on the results, we tested assays that are recommended for LightCycler 1.5 and 2.0 (Roche, Rotkreuz, Switzerland) since these instruments are widely used. The

following real-time PCR assays were selected: Diagenode *Mycoplasma pneumoniae*/*Chlamydophila pneumoniae* real-time PCR (Europe Diagenode sa, Liege, Belgium), GeneProof *Mycoplasma pneumoniae* (GeneProof a.s., Brno, Czech Republic), BactoReal *Mycoplasma pneumoniae* (Ingenetix GmbH, Vienna, Austria) (referred to as Ingenetix hereafter), and LightMix kit *Mycoplasma pneumoniae* (TIB MOLBIOL GmbH, Berlin, Germany). Characteristics of the in-house and commercial kits are summarized in Table 1.

Respiratory samples (34 pharyngeal swabs, 2 sputum samples, and 1 bronchoalveolar lavage sample) were taken between 2011 and 2013 from adult and pediatric patients with symptoms of CAP. Primary testing of samples was done with the repMP1-based real-time PCR. Positive samples ($n = 37$) were aliquoted and frozen at −20°C until retesting with all real-time PCR assays investigated. Amplification of *M. pneumoniae*-specific targets was done in duplicate with each assay, and crossing threshold ($C_T$) values were averaged.

For determination of the kinetics and sensitivity of the real-time PCR assays, a freshly prepared *M. pneumoniae* suspension was homogenized with a 27-gauge syringe to reduce cell aggregations. An aliquot of the suspension was used to determine the number of CFU on PPLO agar with 10-fold dilutions of the bacteria in PPLO bouillon. In parallel, DNA in a further 200-μl aliquot was prepared by using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer (blood and body fluid protocol) and collected in 100 μl elution buffer. DNA concentration was measured in a spectral photometer by the standard procedure. After 10 days of incubation, colonies on PPLO plates were counted. Before use as a sample in real-time PCR, DNA was 10-fold diluted with high-performance liquid chromatography (HPLC)-grade water, aliquoted, and stored at −20°C. PCR efficiency was determined by investigation of a standard curve of the quantified DNA sample diluted over 6 log units, and PCR efficiency of the real-time PCR ap-

**TABLE 1** Characteristics of the in-house and commercial real-time PCR assays investigated

| Assay | LightCycler instrument | Target | Internal control | Sample vol/ test (μl) | Reagent mix | Sensitivity |
|---|---|---|---|---|---|---|
| In-house | 1.5 | repMP1 copies | No | 5 | LC FastStart DNA Master HybProbe (Roche, Mannheim, Germany) | 0.2 CFU[a] |
| Diagenode | 2.0 | P1 adhesin | Yes | 10 | LC TaqMan Master (Roche) | 50 CCU[b] |
| GeneProof | 2.0 | CARDS toxin[c] | Yes | 10 | Included | No information |
| Ingenetix | 1.5 | 16S rRNA | Yes | 5 | LC FastStart DNA Master HybProbe | 0.9 CFU[b] |
| LightMix | 1.5 | P1 adhesin | Yes | 5 | LC FastStart DNA Master HybProbe | 10 copies[b] |

[a] According to Dumke et al. (11).
[b] As specified by the manufacturer.
[c] CARDS toxin, community-acquired respiratory distress syndrome toxin.

proaches was calculated ($10^{1/\text{slope}}$). The sensitivities of the assays were estimated by determining the positivity rate after investigation of three DNA dilutions representing 20, 2, and 0.2 CFU/ reaction, respectively.

To minimize degradation of DNA, none of the samples aliquoted was freeze-thawed more than once before use in real-time PCR. Real-time PCR assays were carried out according to the recommendations of the different manufacturers or as published before (11), and for all runs, the same LightCycler 1.5 or 2.0 instrument was used.

Comparison of results of testing real-time PCR approaches to detect *M. pneumoniae* is complicated. Differences in the target amplified, the special test conditions, and the real-time PCR instrument used can influence the $C_T$ values measured (6). In addition, difficulties with the precise definition of CFU or color-changing units (CCU) in dilutions of the fastidious growing mycoplasmas is a further problem. As a consequence, there are only a limited number of studies that tested more than one *M. pneumoniae*-specific target for quantitative amplification (9–11, 13–15). To our knowledge, beside the in-house repMP1-based approach, only the Diagenode kit was tested previously under comparable conditions with other commercial real-time PCR systems (14).

The standard curves obtained from all five real-time PCR methods tested show excellent statistical characteristics (Table 2). The best PCR efficiency (2.008) was demonstrated with the repMP1-based in-house approach. The lowest efficiency was measured with the Diagenode kit (1.914). Using the in-house assay, a broad range of $C_T$ values from 23.29 to 35.75 was found in respiratory samples of pneumonia patients (Table 3). Since $C_T$ values in respiratory tract samples of at least 20 up to 39 were detected in other reports (10, 11, 13, 15), this range can be considered a typical

spectrum of *M. pneumoniae*-positive specimens in microbiological laboratories. Negative results in the GeneProof, Ingenetix, and LightMix kits correspond to the samples (all were pharyngeal swabs) with the highest $C_T$ values (35.25, 35.27, and 35.75, respectively) detected in the in-house PCR assay. The Diagenode test with these three specimens was positive in one of the two parallel runs, indicating that the detection limit of the procedure was achieved. However, the analytical sensitivity of at least 92% confirmed good performance for all approaches tested. This is in contrast to a study by Touati et al. (14) reporting sensitivities of <90% (88 to 62%) of four of five commercial real-time PCR assays in comparison with an in-house test demonstrating a similar mean $C_T$ value as the repMP1-based assay in the present study. Here, the best results with a commercially available kit were obtained with the Diagenode assay showing a positive test result in all human respiratory samples investigated. Discrepancies of results in comparison to other studies (14) could be explained by differences in the instrument used, concentration of *M. pneumoniae* DNA in samples tested, and sample volume. No amplification was observed with DNA from phylogenetically related or clinically relevant bacterial species *Mycoplasma genitalium* (ATCC 33530), *Mycoplasma hominis* (ATCC 23114), *Mycoplasma orale* (ATCC 23714), *Mycoplasma salivarium* (ATCC 23064), *Ureaplasma urealyticum* (ATCC 27618), *Chlamydophila pneumoniae* (strain TW-183), *Legionella pneumophila* (ATCC 33152), *Haemophilus influenzae* (ATCC 49247), *Streptococcus pneumoniae* (ATCC 6305), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), and human DNA, confirming the specificity of the real-time PCR approaches included.

It should be noted that pretesting of clinical samples with the real-time PCR targeting the repMP1 copies can lead to a selection

**TABLE 2** Kinetics and sensitivity of the evaluated in-house and commercial real-time PCR assays using DNA extracted from bouillon cultures of *M. pneumoniae* M129

| Assay | Standard curve[a] | | % positive samples (mean $C_T$ of positive samples ±SD)[b] | | |
|---|---|---|---|---|---|
| | $r^2$ | PCR efficiency | 52 fg DNA/5 μl (20 CFU/5 μl) | 5.2 fg DNA/5 μl (2 CFU/5 μl) | 0.5 fg DNA/5 μl (0.2 CFU/5 μl) |
| In-house | 0.9994 | 2.008 | 100 (30.55 ± 0.06) | 100 (33.30 ± 0.18) | 100 (34.71 ± 0.67) |
| Diagenode | 0.9995 | 1.914 | 100 (31.76 ± 0.16) | 100 (35.02 ± 0.49) | 75 (38.02 ± 1.19) |
| GeneProof | 0.9988 | 1.994 | 100 (31.07 ± 0.37) | 37.5 (32.96 ± 0.52) | 0 |
| Ingenetix | 0.9999 | 1.938 | 100 (34.60 ± 0.27) | 87.5 (38.35 ± 1.15) | 0 |
| LightMix | 0.9995 | 1.975 | 100 (32.20 ± 0.19) | 100 (36.00 ± 0.93) | 50.0 (37.65 ± 1.17) |

[a] Crossing thresholds ($C_T$s) determined after investigation of 6 dilutions of an *M. pneumoniae* M129 stock (DNA concentration, 169.8 to 0.002 ng/5 μl [$n = 4$ each]).
[b] Investigation of eight parallel runs of DNA of each dilution.

**TABLE 3** Results of four commercial real-time PCR assays used for investigation of 37 *M. pneumoniae*-positive respiratory tract samples compared to the in-house real-time PCR assay

| Assay | No. of positive samples ($n = 37$) (%) | Crossing threshold of positive samples | | | |
|---|---|---|---|---|---|
| | | Mean $\pm$ SD | Range of means | 95% CI[a] | P value[b] |
| In-house | 37 (100) | 30.80 $\pm$ 2.72 | 23.29–35.75 | 29.92–31.68 | |
| Diagenode | 37 (100) | 31.50 $\pm$ 3.28 | 23.29–39.31 | 30.44–32.56 | ns |
| GeneProof | 34 (91.9) | 30.80 $\pm$ 1.98 | 24.44–33.72 | 30.13–31.47 | ns |
| Ingenetix | 35 (94.6) | 34.13 $\pm$ 2.94 | 26.41–40.85 | 33.16–35.10 | <0.001 |
| LightMix | 35 (94.6) | 32.29 $\pm$ 3.05 | 24.32–37.86 | 31.28–33.30 | <0.001 |

[a] 95% CI, 95% confidence interval.
[b] Calculated by paired Student's *t* test (only results of samples positive in both in-house and commercial assay were compared). ns, not significant.

of positive specimens and is a limitation of the present study. However, our results using the repMP1-based system as a sensitive approach to detect *M. pneumoniae* in culture and clinical samples confirmed the findings of previous studies (9, 10, 13). Regarding $C_T$ values, in comparison with the repMP1-based approach, significantly higher mean values were measured with the Ingenetix and LightMix kits. In contrast, the difference to the results of the Diagenode and GeneProof assays is statistically not significant, confirming that under optimized test conditions, monocopy targets can be amplified with efficiency comparable to that of repetitive elements (9). Interestingly, the good clinical sensitivity of the GeneProof kit does not correspond in all cases to the analytical sensitivity. This discrepancy was also reported in other studies (14). Providing that the efficiency of the used DNA preparation corresponds to nearly 100%, all five test kits were able to detect 20 CFU/5 μl of sample in the eight parallel runs tested (Table 2). Whereas the in-house, Diagenode, and LightMix approaches demonstrated positive signals in eight experiments after testing of specimens with 2 CFU/5 μl, amplification products were found in 7 out of 8 (Ingenetix) and 3 out of 8 parallel runs (GeneProof) with the two other kits. Only the repMP1-based approach was able to detect 0.2 CFU/5 μl in all parallel runs.

Results of studies indicated that the bacterial load might influence the severity of disease after infection with *M. pneumoniae* (16, 17). The data of the present and other comparison reports confirmed that the PCR approach used will influence the positivity rate of tested samples and the measured load of *M. pneumoniae* in the specimens. Not only can the mean results for a single sample differ by $C_T$ values of more than 3 for the real-time PCR approaches used (representing a difference of genome copies of around 1 log unit), the mean $C_T$ values for a complete panel of tested samples can also show this range of difference, and this was also reported in other studies (10, 11, 13–15). Furthermore, cultural and molecular detection of *M. pneumoniae* in healthy individuals and long-term carriage of bacteria after the period of typical respiratory symptoms and adequate antibiotic therapy are known facts (18, 19). Future studies have to prove whether a range of bacterial load is associated with this phenomenon and might be used to differentiate patients with acute illness due to *M. pneumoniae* from symptom-less carriers or *M. pneumoniae*-positive patients with symptoms of CAP caused by other microorganisms (20). In this context, knowledge of the sensitivity of the real-time PCR approach used is a precondition for the evaluation of the significance of the concentration of genome copies measured.

In conclusion, with the real-time PCR approaches tested, a rapid (<1.5-h) detection of *M. pneumoniae*-specific targets was demonstrated. Despite differences among the methods regarding

PCR efficiency, $C_T$ values, and analytical and clinical sensitivity, all systems detect at least 20 CFU/5 μl of sample. With regard to the quantitative results of other studies based on real-time PCR (16, 17, 20, 21), this can be considered an acceptable level of test sensitivity for most clinical questions.

## REFERENCES

1. **Waites KB, Talkington DF.** 2004. *Mycoplasma pneumoniae* and its role as a human pathogen. Clin. Microbiol. Rev. **17**:697–728. http://dx.doi.org/10.1128/CMR.17.4.697-728.2004.
2. **Atkinson TP, Balish MF, Waites KB.** 2008. Epidemiology, clinical manifestations, pathogenesis and laboratory detection of *Mycoplasma pneumoniae* infections. FEMS Microbiol. Rev. **32**:956–973. http://dx.doi.org/10.1111/j.1574-6976.2008.00129.x.
3. **Narita M.** 2010. Pathogenesis of extrapulmonary manifestations of *Mycoplasma pneumoniae* infection with special reference to pneumonia. J. Infect. Chemother. **16**:162–169. http://dx.doi.org/10.1007/s10156-010-0044-X.
4. **Atkinson TP, Waites KB.** 2014. *Mycoplasma pneumoniae* infections in childhood. Pediatr. Infect. Dis. J. **33**:92–94. http://dx.doi.org/10.1097/INF.0000000000000171.
5. **von Baum H, Welte T, Marre R, Suttorp N, Lück C, Ewig S.** 2009. *Mycoplasma pneumoniae* pneumonia revisited within the German competence network for community-acquired pneumonia (CAPNETZ). BMC Infect. Dis. **9**:62. http://dx.doi.org/10.1186/1471-2334-9-62.
6. **Loens K, Goossens H, Ieven M.** 2010. Acute respiratory infection due to *Mycoplasma pneumoniae*: current status of diagnostic methods. Eur. J. Clin. Microbiol. Infect. Dis. **29**:1055–1069. http://dx.doi.org/10.1007/s10096-010-0975-2.
7. **Busson L, van den Wijngaert S, Dahma H, Decolvenaer M, di Cesare L, Martin A, Vasseur L, Vandenberg O.** 2013. Evaluation of 10 serological assays for diagnosing *Mycoplasma pneumoniae* infection. Diagn. Microbiol. Infect. Dis. **76**:133–137. http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.027.
8. **Waites KB, Xiao L, Paralanov V, Viscardi RM, Glass JI.** 2012. Molecular methods for the detection of *Mycoplasma* and *Ureaplasma* infections in humans: a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology. J. Mol. Diagn. **14**:437–450. http://dx.doi.org/10.1016/j.jmoldx.2012.06.001.
9. **Schmitt BH, Sloan LM, Patel R.** 2013. Real-time PCR detection of *Mycoplasma pneumoniae* in respiratory specimens. Diagn. Microbiol. Infect. Dis. **77**:202–205. http://dx.doi.org/10.1016/j.diagmicrobio.2013.07.016.
10. **Zhao F, Cao B, He L, Yin Y, Tao X, Song S, Meng F, Zhang J.** 2012. Evaluation of a new real-time PCR assay for detection of *Mycoplasma pneumoniae* in clinical specimens. Biomed. Environ. Sci. **25**:77–81. http://dx.doi.org/10.3967/0895-3988.2012.01.011.
11. **Dumke R, Schurwanz N, Lenz M, Schuppler M, Lück C, Jacobs E.** 2007. Sensitive detection of *Mycoplasma pneumoniae* in human respiratory tract

samples by optimized real-time PCR approach. J. Clin. Microbiol. **45:** 2726–2730. http://dx.doi.org/10.1128/JCM.00321-07.

12. **Dandekar T, Huynen M, Regula JT, Ueberle B, Zimmermann CU, Andrade MA, Doerks T, Sánchez-Pulido L, Snel B, Suyama M, Yuan YP, Herrmann R, Bork P.** 2000. Re-annotating the *Mycoplasma pneumoniae* genome sequence: adding value, function and reading frames. Nucleic Acids Res. **28:**3278–3288. http://dx.doi.org/10.1093/nar/28.17.3278.

13. **Dumke R, Jacobs E.** 2009. Comparison of commercial and in-house real-time PCR assays used for detection of *Mycoplasma pneumoniae*. J. Clin. Microbiol. **47:**441–444. http://dx.doi.org/10.1128/JCM.01989-08.

14. **Touati A, Benard A, Ben Hassen A, Bebear CM, Pereyre S.** 2009. Evaluation of five commercial real-time PCR assays for detection of *Mycoplasma pneumoniae* in respiratory tract specimens. J. Clin. Microbiol. **47:**2269–2271. http://dx.doi.org/10.1128/JCM.00326-09.

15. **Winchell JM, Thurman KA, Mitchell SL, Thacker WL, Fields BS.** 2008. Evaluation of three real-time PCR assays for detection of *Mycoplasma pneumoniae* in an outbreak investigation. J. Clin. Microbiol. **46:**3116–3118. http://dx.doi.org/10.1128/JCM.00440-08.

16. **Nilsson AC, Björkman P, Welinder-Olsson C, Widell A, Persson K.** 2010. Clinical severity of *Mycoplasma pneumoniae* (MP) infection is associated with bacterial load in oropharyngeal secretions but not with MP genotype. BMC Infect. Dis. **10:**39. http://dx.doi.org/10.1186/1471-2334-10-39.

17. **Jiang W, Yan Y, Ji W, Wang Y, Chen Z.** 2014. Clinical significance of different bacterial load of *Mycoplasma pneumoniae* in patients with *Mycoplasma pneumoniae* pneumonia. Braz. J. Infect. Dis. **18:**124–128. http://dx.doi.org/10.1016/j.bjid.2013.06.004.

18. **Gnarpe J, Lundbäck A, Sundelöf B, Gnarpe H.** 1992. Prevalence of *Mycoplasma pneumoniae* in subjectively healthy individuals. Scand. J. Infect. Dis. **24:**161–164. http://dx.doi.org/10.3109/00365549209052607.

19. **Nilsson AC, Björkman P, Persson K.** 2008. Polymerase chain reaction is superior to serology for the diagnosis of acute *Mycoplasma pneumoniae* infection and reveals a high rate of persistent infection. BMC Microbiol. **8:**93–100. http://dx.doi.org/10.1186/1471-2180-8-93.

20. **Spuesens EBM, Fraaij PLA, Visser EG, Hoogenboezem T, Hop WCJ, van Adrichem LNA, Weber F, Moll HA, Broekman B, Berger MY, van Rijsoort-Vos T, van Belkum A, Schutten M, Pas SD, Osterhaus ADME, Hartwig NG, Vink C, van Rossum AMC.** 2013. Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: an observational study. PLoS Med. **10:**e1001444. http://dx.doi.org/10.1371/journal.pmed.1001444.

21. **Spuesens EBM, Meijer A, Bierschenk D, Hoogenboezem T, Donker GA, Hartwig NG, Koopmans MP, Vink C, van Rossum AMC.** 2012. Macrolide resistance determination and molecular typing of *Mycoplasma pneumoniae* in respiratory specimens collected between 1997 and 2008 in The Netherlands. J. Clin. Microbiol. **50:**1999–2004. http://dx.doi.org/10.1128/JCM.00400-12.